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Loss of Color Pigmentation Is Maintained at High Frequency in a Monkey Flower Population

Alex D. Twyford,^{1,2,*} Aaron M. Caola,² Pratibha Choudhary,² Ramesh Raina,² and Jannice Friedman²

1. University of Edinburgh, Institute of Evolutionary Biology, Charlotte Auerbach Road, Edinburgh EH9 3FL, United Kingdom;

2. Department of Biology, Syracuse University, Syracuse, New York 13244

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ABSTRACT: Color polymorphisms have long been of evolutionary interest for their diverse roles, including mate choice, predator avoidance, and pollinator attraction. While color variation is often under strong selection, some taxa demonstrate unexpectedly high frequencies of presumed deleterious color forms. Here we show that a genetic variant underlying complete loss of anthocyanin pigmentation has risen to an unexpectedly high frequency of >0.2 in a natural population of the plant *Mimulus guttatus*. Decreased expression of *MYB5* transcription factor is associated with unpigmented morphs. While the allele was found only in heterozygote adults in the wild, suggesting negative selection, experiments were unable to demonstrate a fitness cost for unpigmented plants, suggesting a cryptic selection pressure in the wild. However, life-history differences among morphs suggests that unpigmented individuals benefit from later flowering and clonal growth. Overall, our study highlights the complex interplay of factors maintaining variation in nature, even for genes of major effect.

Keywords: anthocyanin, genetic variation, *Mimulus guttatus*, polymorphism.

Introduction

The maintenance of polymorphisms at genes of major phenotypic effect has long interested evolutionary biologists. Before modern molecular biology, studying genetic variation in natural populations was limited to visible phenotypes with a known genetic basis, often controlled by one or a few loci. Early theoretical population geneticists used this variation to study the roles of selection and other forces—such as genetic drift, gene flow, and mutation—in maintaining variation within

and between populations (Fisher 1930; Wright 1931; Haldane 1932). Understanding the relative effect of these forces remains a central question in evolutionary biology (Mitchell-Olds et al. 2007; Delph and Kelly 2014). Studying major gene polymorphisms allows researchers to address whether phenotypic convergence involves repeatable genetic changes, whether adaptive mutations are more likely to occur in coding or regulatory regions to assess the relative contributions of adaptive evolution, balancing selection, deleterious variation, and genetic drift in maintaining variation.

Many cases of major gene polymorphisms involve variation in color polymorphism, which can be driven by natural or sexual selection and have profound effects on fitness (e.g., Hoekstra et al. 2006; Rosenblum 2006; Reed et al. 2011). A recent series of articles and discussion (Forsman et al. 2008; Wennersten and Forsman 2012; Bolton et al. 2015, 2016; Forsman 2016) illustrates that the direct and indirect effects of color polymorphism on fitness can be complicated, and whether such polymorphism contributes to population persistence is contentious. Although these articles were focused on animal examples, plants too show dramatic variation in coloration of vegetative and floral structures, and there are many notable cases where variation is present between closely related species (Rausher 2008) or among populations (Sobral et al. 2015) or show segregation within populations (Brown and Clegg 1984).

One of the major classes of plant pigments are anthocyanins, which are a conserved group of phenolic compounds responsible for the pink, red, and blue colors produced by plants. Their conserved nature is also reflected by their conserved genetic basis; flowering plants share seven core enzymes of the anthocyanin biosynthetic pathway (Quattrocchio et al. 1993). Changes or loss of pigment can occur as a result of mutations in core structural genes encoding enzymes or in the transcription factors regulating these genes (Ho and Smith 2016). These colored compounds play diverse adaptive

* Corresponding author; e-mail: alex.twyford@ed.ac.uk.

ORCID: Twyford, <http://orcid.org/0000-0002-8746-6617>; Caola, <http://orcid.org/0000-0002-3110-748X>; Friedman, <http://orcid.org/0000-0002-1146-0892>.

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roles, depending on the tissue where they are expressed. In flowers and fruits they may be involved in pollinator attraction and seed or fruit dispersal, while in vegetative tissues they may have protective roles in response to abiotic and biotic stressors, including ultraviolet (UV) radiation, drought, cold, herbivory, and pathogen defense (reviewed in Strauss and Whittall 2006; Rausher 2008). This tissue-specific regulation is possible due to anthocyanin biosynthetic pathway gene regulation at the transcriptional level by the MYB-bHLH-WD repeat complex (Koes et al. 2005; Davies et al. 2012), and studies show that concentrations of floral and leaf anthocyanins and flavonols are not always correlated (del Valle et al. 2015; Berardi et al. 2016).

Studies in the genus *Mimulus* (monkey flowers) have been informative for understanding plant pigmentation (Wu et al. 2007; Twyford et al. 2015). For example, in their now classic study, Bradshaw and Schemske (2003) show that the shift from bee to hummingbird pollination involved floral pigmentation changes caused by a few genes of major effect. In general, the ~170 monkey flower species show numerous flower color transitions among related species, and the genetic basis of pigmentation in the group has been well studied (e.g., Streisfeld and Rausher 2009; Cooley et al. 2011; Yuan et al. 2016). In a previous common garden experiment of species-wide collections of *Mimulus guttatus*, we noticed a single atypical plant from California lacking red spotting on flowers and having bright green leaves, in contrast to the red-spotted flowers and red-tinged leaves typical of the species (fig. 1). While there are many examples of anthocyanin loss from floral tissue, whole-plant loss of anthocyanin is uncommon in nature (although see Warren and Mackenzie 2001; Wu et al. 2013). Our expectation was that this anthocyanin-less phenotype should be rare in the wild because anthocyanin is present in plants from across the range of *M. guttatus* (and, indeed, widely found across all species of flowering plants; Lawrence et al. 1939), and anthocyanin plays a diverse and important adaptive role. Therefore, a complete loss of anthocyanin is unexpected and an obvious target for removal via purifying selection.

In this study, we pursue our observation of a naturally occurring unpigmented phenotype of *M. guttatus* and use this as an opportunity to investigate the genetics and maintenance of intrapopulation phenotypic variation and the role of anthocyanin for plant performance. We address the following questions: (1) What is the genetic basis of this variation? (2) What is the frequency of this variant in the wild? (3) What are the fitness effects of this phenotype? We address these questions by analyzing phenotypic segregation in experimental crosses and wild-collected seed families, by measuring fitness effects in a common garden study and under experimental conditions where plants are exposed to relevant environmental stresses, and through gene expression analysis. Our results show the complexities of how an in-

triguing major polymorphism underlying an important phenotypic trait can be maintained in a natural population.

Methods

Population Description and Sampling Scheme

In July 2013 we visited the study population in Hume, Sequoia National Forest, California (36°46'58.02"N, 118°53'55.56"W; 5,330 ft above sea level). *Mimulus guttatus* at the site belong to the perennial ecotype. We estimated the census population size at approximately 300 flowering individuals, and no other known *M. guttatus* plants grow within a 3-km range. We phenotyped plants in the field for anthocyanin by scoring for spotting on the flowers (if present) and the visual presence of anthocyanin in the petiole as a proxy for vegetative anthocyanin. We collected open-pollinated seed capsules for segregation analysis, selecting 27 maternal parents each at least 1 m apart to reduce the likelihood of collecting clonal individuals.

Phenotypic Description and Estimates of Allele Frequencies

We grew seed from the 27 field-collected seed families in the greenhouse at Syracuse University, with an average of 21 (range = 6–32) seedlings per family raised to flowering. Seeds were planted in plug trays with Fafard 4P potting mix, stratified at 4°C in the dark for 1 week, and grown at 21°C and a 16L:8D photoperiod in a greenhouse. Flowers and leaves were scored for the presence or absence of pigmentation. We used segregation ratios of these field-collected seed families to infer the maternal genotype and to estimate the frequency of the unpigmented allele in nature (described below).

We confirmed that plants are either absent of anthocyanin or produce undetectable levels in flowers and in leaves by extracting anthocyanin with methanol-HCl from four pigmented and four unpigmented plants and performing thin-layer chromatography in a solvent of BAW, BuHCl, and 1% HCl (Harborne 1998). Pigments were identified on the basis of published retention factors (R_f) in this solvent.

We tested the genetic basis of the anthocyanin phenotype by observing segregation in experimental crosses. We produced two second-generation inbred individuals from single-seed descent, selected for producing progeny that did not segregate for anthocyanin to confirm that they were homozygous. These alternate homozygous parents were intercrossed to produce an F_1 , with anthocyanin scored in the subsequent F_2 's. We raised plants to flowering in the greenhouse and then tested whether the presence/absence of anthocyanin differed from the 3:1 ratio expected from segregation at a single Mendelian locus with a χ^2 test. We determined whether unpigmented plants are plastic for anthocyanin production by exposing them to a high-light-intensity stressful environment that typically induces anthocyanin (Albert et al. 2009). We

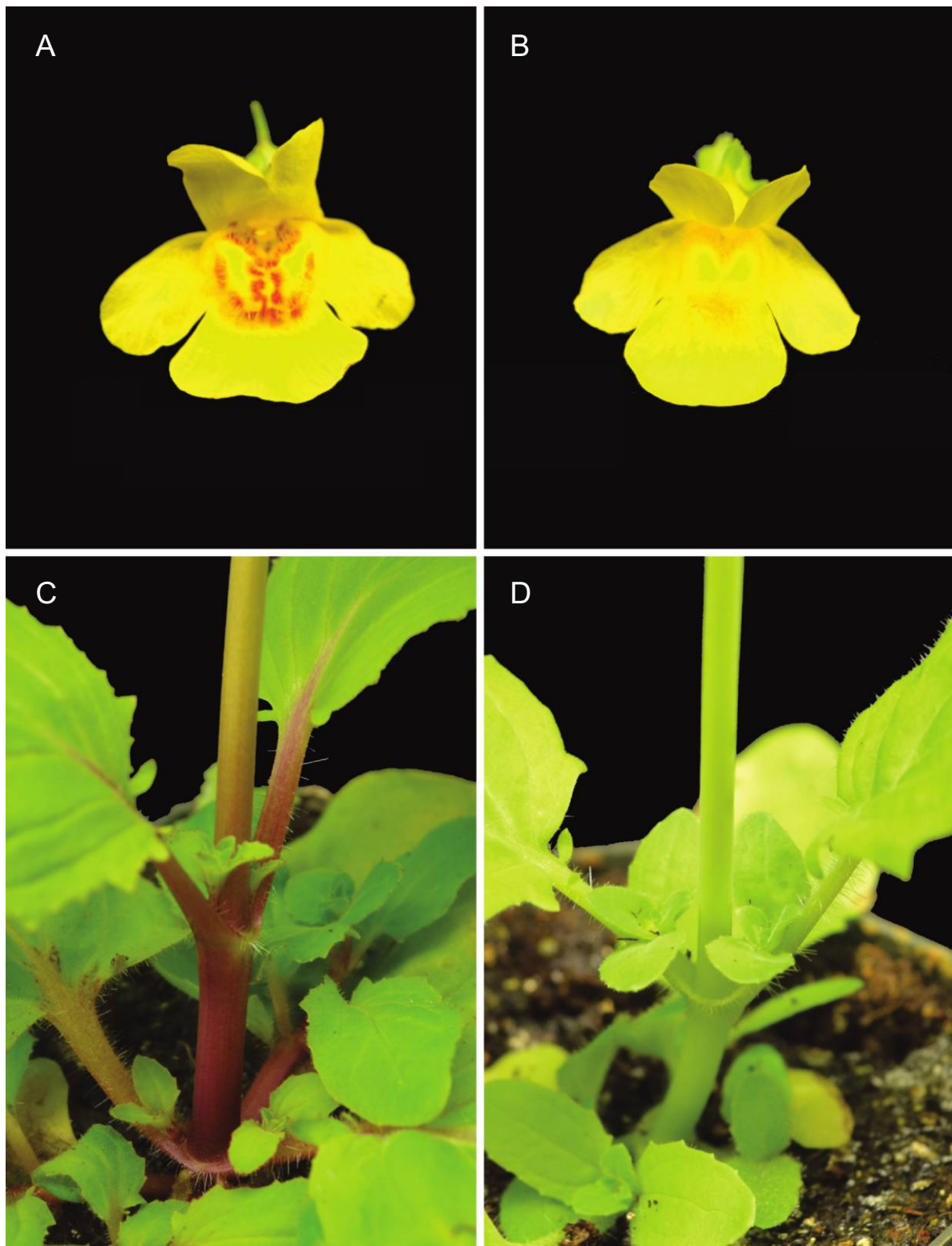


Figure 1: Photographs of representative *Mimulus guttatus*. Pigmented (AA; A) and anthocyanin-deficient (aa; B) flowers and pigmented (C) and anthocyanin-deficient (D) stems and leaves.

recorded the presence or absence of anthocyanin after 1 week of exposure to natural UV light conditions and drought on the roof of the Life Science Complex at Syracuse University in a set of 48 unpigmented and 48 wild-type F_2 plants.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The R2R3 MYB transcription factors are regulators of anthocyanin biosynthesis in a wide range of plant species (Liu et al. 2015). Recently, five R2R3-MYB genes were identified in *M. guttatus* (Cooley et al. 2011) and thus are good candidates for regulators of anthocyanin. To test whether any of these MYB genes regulate anthocyanin accumulation, we examined the transcript levels of *MgMYB1-5* in the leaves and floral buds of AA, Aa, and aa plants. We collected 2-week-old leaves and unopened floral buds from four plants of each genotype and pooled them for RNA isolation. We isolated total RNA using TRIzol reagent (Invitrogen) as per manufacturer's instructions and determined RNA concentration on agarose gel and by spectrophotometer (Bio-Rad). We retrieved *MgMYB1-MgMYB5* gene sequences from <http://www.phytozome.net> and designed appropriate primers. Details of primer sequences, cDNA synthesis, and RT-PCR reactions are given in the appendix, available online. We used *M. guttatus* UBIQUITIN-CONJUGATING ENZYME (UBC) gene as a normalization control (Yuan et al. 2014). As a positive control for PCR, we used genomic DNA isolated using Edward's protocol (Edward et al. 1991).

Measures of Fitness

We tested whether the absence of anthocyanin affects vigor, pollination, and seed set between genotypic classes when grown outside in an experimental field. Initially, we identified 10 unrelated homozygous wild-type and seven unrelated homozygous mutant plants by selfing plants from the segregating families of field-collected plants and retaining only those parents that did not produce any segregating progeny. These plants then served as the parents for our crossing scheme. Plants were crossed in a round-robin design to generate outcrossed individuals of three genotypic classes (outcrossed pigmented [six crosses], outcrossed unpigmented [six crosses], pigmented \times unpigmented [six crosses]). In 2014, a total of 116 individuals consisting of six to seven full-sib individuals from each of these 18 crosses were planted in a randomized block design in an experimental field site at Syracuse University. We measured a suite of vegetative and flowering traits at 3-week intervals: plant height and width, number of stolons, and number of flowering branches. In addition, we recorded leaf length (3 weeks after transplant) and the date of first flower, and we ranked the plants for senescence on a five-point scale at the end of the season. Flowers were available

to be visited by pollinators, and we collected at least two seed capsules from each plant before dehiscing and counted the number of seeds per capsule on a random subset of 40 plants. We provided plants with daily supplemental water until all plants were finishing flowering. The following spring we assessed over-winter survival by recording any plants with signs of regrowth.

We conducted two separate growth chamber experiments, where we investigated the effect of exposure to UV light and the effect of drought conditions. For each experiment, we used a total of 12 crosses (four of each genotype) with eight full-sibs per family. In both experiments, 192 plants were grown for 10 weeks in a growth chamber with a 16L:8D photoperiod at 21° and 18°C. In the UV experiment, half of the plants were assigned to a control treatment with regular light (PAR = 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$, UV = 10 $\mu\text{W cm}^{-2}$), and the other half were assigned to a UV treatment with supplemental UV light (PAR = 258 $\mu\text{mol m}^{-2} \text{s}^{-1}$, UV = 2,360 $\mu\text{W cm}^{-2}$). In the second experiment, half the plants were watered with our regular regimen of soaking flats for 1 h daily, while the other plants were watered every 4 days. For both experiments, we measured plants for germination timing and a suite of growth, flowering, and senescence traits at regular intervals. All phenotypic data are deposited in Dryad (Twyford et al. 2018).

We analyzed the fitness experiments with REML general linear models in SAS (mixed procedure, release 9.4; SAS Institute 2015). Within each experiment, dependent variables were standardized to a mean of 0 and standard deviation of 1 (to facilitate comparisons between experiments). For the field experiments, analyses included the effect of genotype as a fixed categorical factor and block and family nested with genotype as random factors. The significance of family was assessed using a log-likelihood ratio test and a χ^2 test statistic (Littell et al. 1996). For the UV and drought experiments, we used similar models and also included the fixed effect of treatment and its interaction with genotype. We analyzed pairwise significant fixed effects with Tukey's method, which adjusts the type I error rate for multiple testing.

Results

Phenotypic Observations

Our greenhouse observations made on all 568 individuals from 27 wild-collected seed families used across experiments revealed two discrete classes of pigmentation, with either individuals having anthocyanin or plants being entirely unpigmented (fig. 1). Thin-layer chromatographic analysis of floral and leaf extracts from four pigmented and four unpigmented plants confirmed that unpigmented plants do not produce detectable levels of pigment with a retention factor (R_f) in the range expected of anthocyanin, in contrast to the

pigmented plants. We observed that 48 unpigmented greenhouse-grown plants—which were then exposed to natural UV light conditions and drought on the Syracuse University rooftop—still did not produce visible anthocyanin (results not shown). These results show that the unpigmented phenotype is genetic and can reliably be scored as a simple binary trait under glasshouse conditions.

Genetic Basis

We used segregation in an F_2 family derived from a cross between an inbred pigmented and an inbred unpigmented plant to investigate the genetic basis of the phenotype. The F_2 progeny segregated in a ratio of 108:29 pigmented:unpigmented, which did not significantly differ from the 3:1 ratio expected from simple Mendelian segregation ($\chi^2 = 1.073$, $P = .3$). There was perfect cosegregation between floral and vegetative anthocyanins in the progeny and in wild-collected families (below), suggesting that loss of anthocyanin in flowers and vegetative tissue is controlled by a single locus. Because three-quarters of F_2 's and all F_1 plants produce anthocyanin, the presence of anthocyanin is dominant. As such, the unpigmented phenotype is controlled by a single allele a , which is recessive to the wild-type allele A .

Transcription of *MYB1-5* Genes

We examined transcript levels of *MYB1-5* in the leaves and floral buds of AA , Aa , and aa plants to test for differences among genotypes. Only *MYB4* and *MYB5* transcripts were detected in the leaves and floral buds of AA , Aa , and aa plants, consistent with the previous report of not detecting transcripts of *MYB1-3* in the floral buds of an inbred line (IM767) of *Mimulus guttatus* (Yuan et al. 2014). Level of *MYB4* transcript was similar in the leaves and floral buds of all three genotypes. Only *MYB5* transcript was differentially expressed in the three genotypes for both leaves and floral buds (fig. 2A). Transcript levels were highest in AA plants and lowest in aa plants (fig. 2A). This correlates with the visible level of anthocyanin accumulation in these plants: highest in the AA and lowest in aa plants (fig. 1). Taken together, our results suggest that *MYB5* positively regulates anthocyanin accumulation in *M. guttatus*, and decreased expression is associated with the anthocyanin deficient phenotype.

Allele Frequencies in the Wild

We estimated the frequency of the anthocyanin allele in the wild using information from the phenotypic class of the parent scored in nature and the progeny segregation ratios. All plants phenotyped in the wild produced anthocyanin and thus are genotype AA or Aa . Eleven of the 27 seed families (40%) raised from these plants subsequently segregated for pig-

mentation (mean proportion unpigmented = 0.23; range = 0.06–0.36; see table A2; tables A1, A2 are available online), and thus the maternal parent has genotype Aa . The remaining 16 families did not segregate for anthocyanin production. These may have maternal parents with genotype AA , or genotype Aa where no homozygous recessive progeny are present because of the preponderance of the dominant A allele in the pollen pool or because of small sample sizes for some families. Assuming that these parents with no segregating offspring are AA gives a conservative lower estimate of 0.2 for the a allele (i.e., 11 Aa and 16 AA , frequency of a is 11/54). To give an upper estimate on the frequency of a , if all non-segregating plants were actually heterozygous (i.e., 27 Aa), the frequency of a would be 0.5.

Measures of Fitness

In our experimental field common garden with 116 outcrossed individuals of known genotype, the genotypes differed in some growth measures. They did not differ in leaf length (3 weeks after transplant) or seed set (table 1). Unpigmented plants flowered later (mean days to flower: AA , 51.6 ± 0.9 ; Aa , 53.02 ± 1.03 ; aa , 61.0 ± 1.05) and made fewer flowering branches (mean branches: AA , 5.0 ± 0.49 ; Aa , 5.3 ± 0.5 ; aa , 2.6 ± 0.43) but made significantly more stolons (mean stolons: AA , 1.6 ± 0.26 ; Aa , 2.9 ± 0.32 ; aa , 5.8 ± 0.27 ; fig. 3). For all traits except stolon production, the AA and Aa genotypes did not differ significantly from one another. Nonetheless, when we calculate gene action of each allele at the single locus, all traits show either partial dominance or overdominance (table 1).

In the growth chamber experiments with either supplementary UV light or drought treatments, we found that similar to the field experiment, genotypes differed significantly for most traits, driven by differences between the aa genotype and the other two genotypes (table 1). We expected aa genotypes to be most affected by the stressors; however, this was not borne out. Within an experiment, the genotypes generally responded similarly to the stressors, for example, by flowering significantly later under UV and significantly earlier under drought (fig. 3A). In both experiments, there was a significant genotype \times treatment interaction for stolons. Plants of aa genotype increased their stolon production in both UV and drought, while the other genotypes either decreased stolons or did not change significantly from control (fig. 3B).

Discussion

We have described the genetic basis, frequency, and fitness effects of a segregating color polymorphism maintained in a natural population. The absence of anthocyanin in some plants of the Hume population of *Mimulus guttatus* is con-

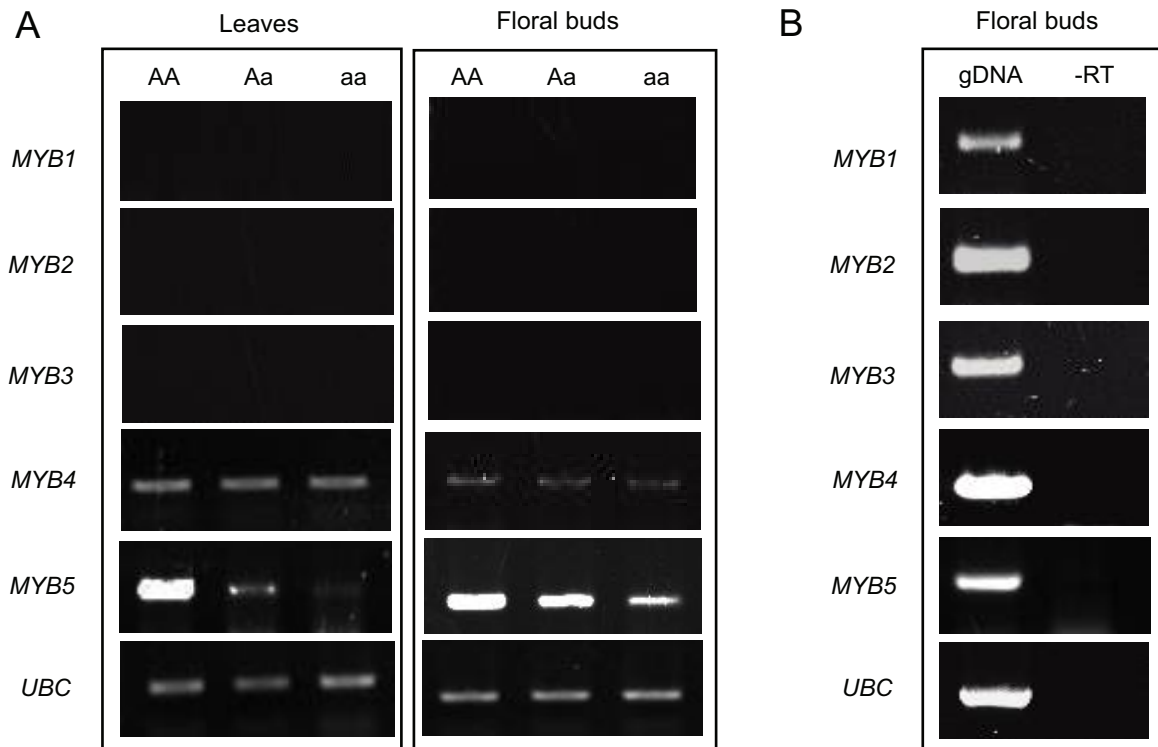


Figure 2: Gene expression analysis of *R2R3-MYB* genes in *Mimulus guttatus*. *A*, Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of *MYB1-5* genes in leaves and floral buds of plants with genotype *AA*, *Aa*, and *aa*. For RNA isolation, leaves were pooled from four plants of each genotype. *UBC* was used as a normalization control for RNA concentration. *B*, Genomic DNA was used as a positive control, and $-RT$ (reaction without reverse transcriptase) was used as a negative control in RT-PCR experiments. Experiments were repeated with two independent biological samples of leaves and floral buds with similar results. PCR primers and cycle numbers are given in the appendix, available online.

trolled by a single recessive allele, which negatively affects expression of the *MYB5* transcription factor. The causal allele is present at a surprisingly high frequency of >0.2 in this population. Our experiments did not identify a cost of this allele on attractiveness to pollinators or on survival in a field environment, and plants with this phenotype performed well in response to environmental stressors, including high UV light and drought. One clue to how this variant is maintained may come from the close association between the recessive phenotype and an alternative life-history strategy: unpigmented plants consistently flowered later and invested more in stolons. We consider below how these results improve our understanding of the maintenance of phenotypic variation.

The unpigmented *M. guttatus* phenotype represents a natural variant where anthocyanin is not produced in any tissue of the plant, and crosses revealed that floral and vegetative anthocyanin always cosegregate. This is unusual because most plants with mutations for unpigmented flower variants still produce some anthocyanin in stems and leaves (Warren and Mackenzie 2001), or the loss of anthocyanin occurs indepen-

dently in flowers and vegetative tissues (e.g., Dick et al. 2011). However, estimating the occurrence of complete loss of anthocyanin phenotypes is challenging because many studies report the presence of pigment only in flowers and not in vegetative tissues, or they do not discriminate between reduced pigment levels and the absence of pigmentation.

Selection Pressures and the Maintenance of Variation

The maintenance of major gene polymorphisms within a single population are intriguing, especially cases such as this, where a presumably deleterious allele has risen to a relatively high frequency (>0.2). This frequency is substantially higher than many other loss-of anthocyanin mutations that segregate within populations (e.g., *Mimulus lewisii*, 0.03; Wu et al. 2013; *Ipomoea purpurea*, 0.005; Coberly and Rausher 2003). To date, most empirical studies in animals suggest that such polymorphisms are maintained by negative frequency-dependent selection, mediated by apostatic or sexual selection (Gray and McKinnon 2007). We cannot see a scenario in which the unpigmented and pigmented plants experience a selective

Table 1: Summary of restricted maximum likelihood mixed effect models on morphological traits in field experiment, growth chamber experiment with two treatments (control and ultraviolet [UV] light), and growth chamber experiment with two treatments (control and drought)

Source of variation	Germination	Flowering time	Leaf length	Stolons	Flowering branches	Seed set
Field experiment:						
Genotype		$F_{2,101} = 18.82^{***}$	$F_{2,107} = 1.06$	$F_{2,92.8} = 61.65^{***}$	$F_{2,92} = 8.92^{**}$	$F_{2,11.54} = .89$
AA vs. Aa		$t_{101} = .86$		$t_{92.1} = 3.09^{**}$	$t_{92} = 1.26$	
AA vs. aa		$t_{101} = 5.73^{***}$		$t_{93.2} = 10.70^{***}$	$t_{92} = 2.74^*$	
Aa vs. aa		$t_{101} = 4.80^{***}$		$t_{93} = 7.37^{***}$	$t_{92} = 4.08^{**}$	
Family (genotype)		$\chi^2 = .7$	$\chi^2 = 1.7$	$\chi^2 = 1.1$	$\chi^2 = 0$	$\chi^2 = 0$
UV experiment:						
Genotype	$F_{2,185} = 3.87^*$	$F_{2,9.04} = 8.91^{**}$	$F_{2,9.09} = 1.12$	$F_{2,8.88} = 4.72^*$	$F_{2,9.18} = .42$	
AA vs. Aa	$t_{185} = .54$	$t_{8.95} = .72$		$t_{8.85} = 1.57$		
AA vs. aa	$t_{187} = 2.63^*$	$t_{9.13} = 3.96^*$		$t_{8.91} = 3.07^*$		
Aa vs. aa	$t_{185} = 2.15^*$	$t_{9.03} = 3.26^*$		$t_{8.87} = 1.50$		
Treatment	NA	$F_{1,3.84} = 9.82^*$	$F_{1,3.79} = 10.61^*$	$F_{1,3.95} = .93$	$F_{1,3.93} = .71$	
Genotype × treatment			$F_{2,174} = 7.07^{**}$	$F_{2,170} = 6.12^{**}$		
Family (genotype)	$\chi^2 = 0$	$\chi^2 = 15.2^{***}$	$\chi^2 = 16.8^{***}$	$\chi^2 = 21.9^{***}$	$\chi^2 = 39.2^{***}$	
Drought experiment:						
Genotype	$F_{2,9} = .48$	$F_{2,9.18} = 13.32^{**}$	$F_{2,9.14} = 13.09^{**}$	$F_{2,182} = 12.96^{***}$	$F_{2,9.22} = 11.31^*$	
AA vs. Aa		$t_{9.13} = .39$	$t_{9.02} = 1.35$	$t_{181} = 1.73$	$t_{9.12} = 2.64$	
AA vs. aa		$t_{9.29} = 4.26^*$	$t_{9.26} = 3.59^*$	$t_{182} = 5.01^{***}$	$t_{9.33} = 6.13^{***}$	
Aa vs. aa		$t_{9.13} = 4.66^{**}$	$t_{9.12} = 4.96^{**}$	$t_{182} = 3.31^{**}$	$t_{9.18} = 3.52^*$	
Treatment		$F_{1,3.72} = 16.56^*$	$F_{1,1.69} = 17.50^{***}$	$F_{1,3.98} = .17$	$F_{1,3.9} = 18.91^{***}$	
Genotype × treatment				$F_{2,182} = 8.47^{**}$		
Family (genotype)	$\chi^2 = 1.6$	$\chi^2 = 10.2^{**}$	$\chi^2 = 7.4^*$	$\chi^2 = 1.8$	$\chi^2 = 15.16^{***}$	
Gene action:						
A		4.71	.04	4.71	1.21	23.38
D		3.29	.42	3.29	1.98	10.70
d/a		.70	11.5	.70	1.65	.46

Note: Plants were of known genotype for anthocyanin production. If the effect of genotype is significant, we present pairwise comparisons. Germination was not measured in the field experiment, and seed set was measured only in the field experiment. Magnitude of additive (A) and dominant (D) gene action in the field experiment for the two alleles at the single anthocyanin locus was calculated on nonstandardized data. NA, not applicable.

* $P < .05$.

** $P < .001$.

*** $P < .0001$.

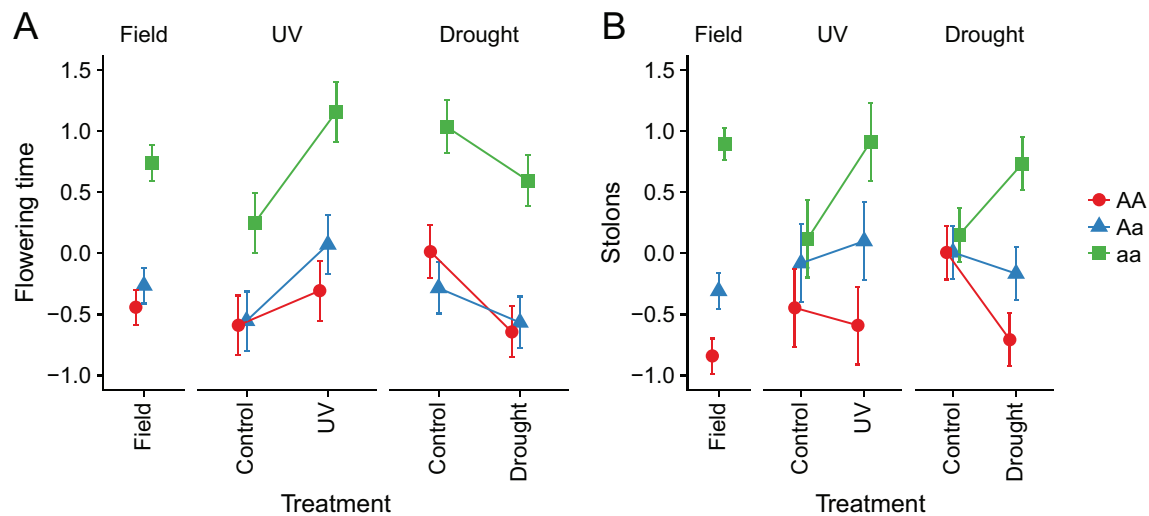


Figure 3: Influences of genotype and treatment on flowering time and stolon number. Flowering time (A) and number of stolons (B) in three experiments (field, ultraviolet [UV] treatment, drought treatment) with plants of known anthocyanin genotype. Standardized means \pm SE data are shown. For statistical details, see table 1.

advantage when rare, and so we do not think that this is the mechanism responsible for its maintenance. We can also cast doubt on some other possible explanations, such as genetic drift, mutation-selection balance, or heterozygote advantage. Random genetic drift could be causal and has been implicated in segregating color polymorphisms in northern leopard frogs (Hoffman et al. 2006) and the candy-stripe spider (Oxford 2005). In the latter example, the rare morph occurs at low frequencies (~ 0.05 – 0.3) in small populations, and the authors suggest that selection is weak and drift dominates. Although intermittent drift (Oxford and Shaw 1986) may be involved in this *M. guttatus* population, the correlated phenotypic effects of anthocyanin on other traits (discussed below) suggest that genetic drift is unlikely the main mechanism. We also believe mutation-selection balance is unlikely because we did not find complementary gene action when performing crosses among different families of unpigmented plants (i.e., offspring were unpigmented, suggesting that the causal mutation is in a single gene). It seems improbable that a mutation keeps arising in the same gene within this population. There is some indication of heterozygote advantage, as we find overdominance for some traits (height [not shown], leaf length, flowering stems; table 1), but it's unclear whether these traits result in higher fitness. Thus, we are reluctant to suggest that the polymorphism is maintained by overdominance at this single locus in a single population (simple overdominance; Delph and Kelly 2014), especially given how rare this phenomenon is in nature (although see Tuttle 2003; Johnston et al. 2013).

A probable explanation for the maintenance of the polymorphism is that selection is acting on suites of traits that include the pigment phenotype rather than the pigment trait

itself. Our fitness and growth experiments show that homozygote recessives (*aa*) make more stolons (clonal growth) flower later and for longer. Overall, the *aa* genotype has a more perennial life-history strategy, investing more in vegetative growth with delayed flowering (Friedman et al. 2015). This finding is not unique to *Mimulus*; indeed, many organisms demonstrating major polymorphism exhibit true alternative life-history strategies (Tuttle 2003; Silva et al. 2015). As such, selection may be acting on life-history traits, with a more clonal phenotype being advantageous in this permanently wet site that facilitates late-season flowering and persistence via clonal reproduction. This scenario would entail pleiotropic gene effects or physical linkage between life-history traits and the pigmentation gene (discussed below). Thus, it is possible that clonality may be involved in the maintenance of the unpigmented plants, particularly if the population was founded by a small number of individuals. To assess whether selection is acting through stolon production would require measuring lifetime fitness in the field for this perennial plant and assessing fitness components through survival, stolons, and seed production.

We also cannot exclude the possibility that the maintenance of the phenotype is due to complex selection pressures that occur in the wild, including fine-scale spatial and temporal differences in selection, potentially in conjunction with selection on cryptic trait variation. This seems plausible because even systems with intensely studied color polymorphisms often fail to locate the target of selection (reviewed in Gray and McKinnon 2007). This issue may be particularly problematic with pigments such as anthocyanins that play extremely diverse functions and where fitness in an experimental field may fail to capture components of selection in

the wild (Waser and Price 1981). We found that unpigmented plants had equivalent seed set to pigmented plants in a field setting, and if this was also the case in the native environment, it would suggest that the generalist pollinators that visit *M. guttatus* do not discriminate against plants with subtle shifts in flower color from yellow with red spots to unspotted flowers. Similarly, our growth experiments showed that unpigmented plants grow well under field conditions as well as in growth chamber experiments with elevated UV light and drought conditions. These findings are consistent with *Arabidopsis* anthocyanin mutants that had equivalent fitness to wild-type plants under stressful conditions (von Wettberg et al. 2010), although *Petunia* mutants deficient in *F3'H* grew more slowly under UVB than wild-type plants (Ryan et al. 2002). One obvious pressure we did not directly investigate is herbivory. Anthocyanin strongly affects the preference of many herbivores (Strauss and Whittall 2006), and herbivory tests with slugs and Lepidoptera show a preference for nonpigmented over pigmented flowers in wild radishes (*Raphanus sativus*; Irwin et al. 2003). We also have not investigated whether loss of anthocyanin has benefits in terms of resource expenditure, particularly in limiting environments.

Genetic Basis

Loss of anthocyanin can occur because of mutations in structural genes or in transcription factors that regulate the expression of structural genes. While loss of anthocyanin from all plant tissues is usually attributed to mutations in structural genes, *MYB5* appears to be a candidate transcription factor that regulates anthocyanin expression across the plant. Our gene expression analysis found that *MYB5* was the only differentially expressed candidate transcription factor consistent with the observed phenotypes. The role of this transcription factor is supported by Yuan et al. (2014), who showed that *MYB5* is the only anthocyanin activating *R2R3-MYB* transcription factor (sensu Cooley et al. 2011) expressed in the corolla of *M. guttatus*. In the related *Mimulus lewisii*, mutations in *MIWD40a* (part of the *MYB-bHLH-WD40* regulatory complex) affected anthocyanin accumulation in both flowers and stems, while mutations in *R2R3-MYBs*, including those most closely related to *MgMYB5*, affected only floral anthocyanin. Similarly, in *Mimulus aurantiacus*, *MaMYB2* (most closely related to *MgMYB5*) is necessary for floral anthocyanin pigmentation (Streisfeld and Rausher 2009; Streisfeld et al. 2013). Finally, Lowry et al. (2012) show that variation among populations of *M. guttatus* in leaf and floral anthocyanin can be ascribed to an *R2R3-MYB* cluster (but not including *MYB5*); however, their study deals with differences in anthocyanin intensity and pattern and not presence/absence. While we have identified the likely involvement of *MYB5*, further work will be required to prove its role as

the causal gene and to understand the molecular mechanism underlying the phenotype. This could be tested by determining the expression of genes in families segregating for the anthocyanin phenotype or by using recently developed transformational protocols for *M. guttatus* (Preston et al. 2014).

Our hypothesis that natural selection is not acting directly on anthocyanin genes but on correlated life-history traits raises the prospect of physical linkage between (a regulator of) *MYB5* and life-history traits. While there are many candidate genes underlying the perennial strategy in *M. guttatus*, the most obvious is the large *DIV1* inversion that has a major phenotypic effect (Lowry and Willis 2010). Although this inversion contains a tandem *MYB* array responsible for some anthocyanin phenotypes in *M. guttatus* (Lowry et al. 2012), it is not the location of the *MYB5* locus that has reduced expression. *MYB5* is on linkage group 12, at position 6,076,089 (Migut.L00458; <https://phytozome.jgi.doe.gov>). There are not many candidate genes in this region, but a gene showing homology to *PROTEIN SUPPRESSOR OF PHYA-105 (SPA1)* is located about 1 MB away at 7,216,182 (Migut.L00551). *SPA1* is known to negatively regulate anthocyanin accumulation, plant size, and flowering in *Arabidopsis*, especially in short days (Ishikawa et al. 2006). Hypothetically, an accumulation of *SPA1* in *aa* plants might explain its decreased anthocyanin accumulation, bigger vegetative growth, and delayed flowering phenotypes. Future work will be necessary to test these hypotheses.

Conclusions

The maintenance of multiple variants within a population provides a rare opportunity to explore allele dynamics and phenotypic variation within a shared environmental context. Although we have been unable to identify the ecological mechanisms favoring the color polymorphism in this population, the high allele frequency suggests that either there is selection for the color variant or it is favored via pleiotropic effects or linkage disequilibrium with other traits under selection. We suggest that this provides an opportunity to examine the interconnections between evolutionary processes, such as different forms of selection and drift, and to link ecological mechanisms favoring polymorphisms with their genetic basis.

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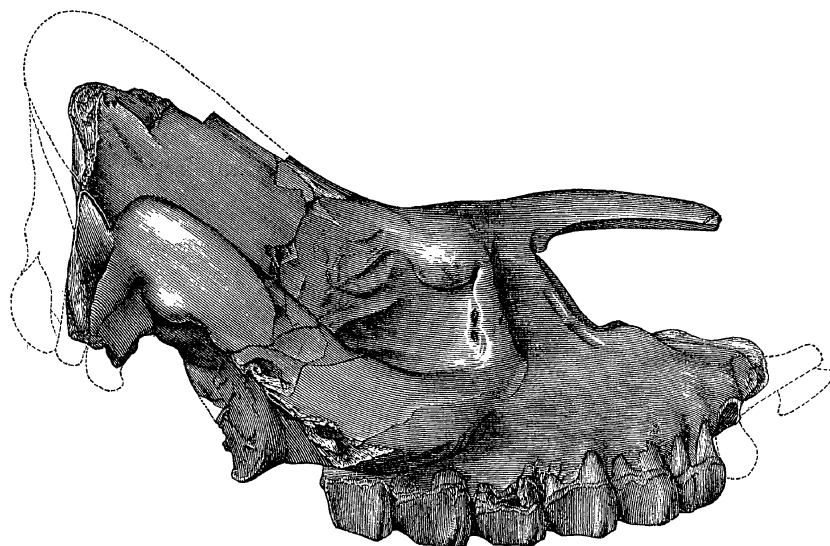
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"The *A[phelops] malacorhinus* is a comparatively long-limbed animal, and its apparent elevation was increased by the shortness of the body, and especially of the neck. There was probably a great development of the upper lip, or snout, and the face was concave in profile." From "On the Extinct American Rhinoceroses and their Allies" by E. D. Cope (*The American Naturalist*, 1879, 13:771a–771j).